Ferritin Crystal Cataracts in Hereditary Hyperferritinemia Cataract Syndrome


PURPOSE. Hereditary hyperferritinemia cataract syndrome (HHCS) is a genetic disease defined by cataracts, hyperferritinemia, and ferritin light-chain (L-ferritin) gene mutations. HHCS was diagnosed in this study in one of the first families known to be affected in the United States, and the basis of lens opacities in HHCS was determined.

METHODS. DNA amplification and sequencing of the human L-ferritin gene was used for mutation detection. RNA electrophoretic mobility shift assay was performed to demonstrate functional consequences of a new mutation. Opacities were characterized by immunohistochemical and electron microscopic analyses of human HHCS lens aspirate.

RESULTS. HHCS was diagnosed in five members of one family who had all three hallmark features: hyperferritinemia, a prominent cataract by history, and the finding of a novel mutation in the L-ferritin gene (C33T). This mutation interferes with function of the L-ferritin transcript in an RNA gel shift assay. Light-diffracting crystalline deposits were present in cataractous lenses from two affected family members but not in control lenses. Immunohistochemical analysis showed strong anti-L-ferritin reactivity in the crystalline deposits. Analysis of these deposits by transmission electron microscopy with fast Fourier transformation demonstrated macromolecular crystalline structure of the deposits. The data were consistent with a face-centered cubic crystal having a unit crystal cell size of 17 nm, both findings characteristic of ferritin crystals grown in vitro.

CONCLUSIONS. HHCS cataract is due to numerous small opacities, predominantly in the lens cortex, that are light-diffracting ferritin crystals. Patients with HHCS may be recognized by a family history of cataracts and hyperferritinemia without increased serum iron. (Invest Ophtalmol Vis Sci. 2002;43: 1121–1126)

Cataracts are the predominant clinical abnormality in the recently described genetic disease hereditary hyperferritinemia cataract syndrome (HHCS). HHCS has been identified in patients who had both high serum ferritin (hyperferritinemia) and a prominent history of bilateral, early-onset cataract. It is inherited in an autosomal dominant manner and results from mutations that cluster in a discrete regulatory region of the human L-ferritin gene, as discussed later in the article. A 5- to 20-fold increase in ferritin is found in serum, lymphocytes, liver, aqueous humor and lens of patients with HHCS. Recent data indicate that patients with HHCS have ferritin accumulations in other tissues and that this may have pathologic consequences (Brooks D, Stambolian D, manuscript in preparation).

HHCS is caused by a novel mechanism involving loss of the normal regulation of ferritin gene expression. The mechanism by which iron regulates ferritin synthesis is a paradigm of translational regulation of gene expression (reviewed in Ref. 6). The two essential components of this regulatory system are a cis-acting iron-responsive element (IRE) and trans-acting iron-regulatory proteins (IRPs) as shown in Figure 1. The IRE in ferritin mRNAs is a hairpinlike structure near the beginning of the 5' untranslated region (UTR). IRPs bind this RNA element and decrease ferritin translation by competing for ribosome binding. An increase in iron decreases IRP binding, relieving inhibition of ferritin mRNA translation (Fig. 1, middle panel), resulting in increased ferritin storage of iron. This iron-dependent regulation of ferritin expression was observed in vitro, but its significance was confirmed by the identification of HHCS, in which deregulation of IRE-mediated regulation of ferritin synthesis leads to persistent hyperferritinemia (Fig. 1, right panel).

Ferritin is found in the lens, where it may have a role in iron homeostasis, as it does in other tissues. Assembly of L (light chain) and H (heavy chain) ferritin subunits into a 24-subunit complex yields apoferritin. Apoferritin is a cage-like structure capable of containing up to 4000 atoms of iron as holoferritin. H-ferritin translocates and oxidizes ferrous iron inside the ferritin cage. L-ferritin, which does not bind iron directly, facilitates mineralization of ferric iron inside the cage to form holoferritin. The coordinated action of L- and H-ferritin subunits detoxifies ferric iron inside the holoferritin cage, thereby limiting redox cycling of iron and generation of reactive oxygen species. These functions of ferritin, iron storage, and detoxification, did not provide an obvious hypothesis connecting overexpression of L-ferritin to cataract. The present study was undertaken to diagnose HHCS and determine the cause of cataract in this disorder.

Cataracts may appear as early as infancy in HHCS and have been described as numerous, pulverulent (dustlike) opacities of the lens that cause glare and decrease visual acuity. Two groups have reported a 5- to 10-fold higher L-ferritin content in HHCS lens and/or aqueous humor than in non-HHCS eyes. However, their findings differed regarding the form of L-ferritin in lens. One group reported only soluble ferritin, whereas another group suggested that L-ferritin forms crystalline-appearing deposits in the lens. Herein, we report a novel mutation of the L-ferritin IRE and demonstrate that it has functional consequences that result in HHCS. Comprehensive evaluation of lens specimens removed during cataract surgery demonstrates that light-diffracting crystals of L-ferritin-rich isoferritin are present in the lens of patients with HHCS. We argue that...
these crystals are one type of opacity causing cataract in HHCS and an unprecedented example of a wild-type protein’s crystallizing in human lens.

**METHODS**

**Family Data**

A 50-year-old woman sought treatment at the Hemochromatosis Clinic at the University of Pennsylvania because of a personal and family history of hyperferritinemia. Medical history included stable ferritin levels of 1300 to 1440 ng/mL (normal: 50–200) with normal serum iron, transferrin, transferrin saturation, and HFE-1 mutation testing. Bilateral cataracts were diagnosed at 5 years of age; progressive glare prompted bilateral cataract surgery in her fourth decade. This proband and five family members were subjected to ophthalmic examination, serum ferritin assay, and human L-ferritin IRE genotyping. Four additional family members were found to have hyperferritinemia in the range of 969 to 1350 ng/mL. Each of these individuals also exhibited cataracts—numerous small opacities, predominantly in the cortex of each lens. One affected family member had received a previous misdiagnosis of hemochromatosis and prescribed phlebotomy therapy. Removal of 2 units of blood over 3 weeks induced symptomatic iron-deficiency anemia, a characteristic response in patients with HHCS to inappropriate phlebotomy. Two family members with normal serum ferritin had clear lenses. The one who was available for genetic testing had a normal L-ferritin IRE DNA sequence. All patients signed informed consent. This research protocol was approved by the Institutional Review Board of the University of Pennsylvania and conformed to the tenets of the Declaration of Helsinki.

**DNA Sequence Analysis**

Peripheral blood leukocyte genomic DNA was prepared from individuals with hyperferritinemia who had cataract, and control DNA was collected from 65 cataract-free individuals (130 chromosomes). The human L-ferritin gene was subjected to PCR amplification, using oligonucleotides and conditions as described in Girelli et al. PCR amplicons and individual subclones were sequenced by dideoxy-termination (ABI 377 sequencer; PB-Applied Biosystems, Foster, CA).
Human Lens Preparation

Two affected individuals had cataract surgery that produced a disrupted lens aspirate in saline suspension. This aspirate was centrifuged, and the pellet was examined directly (untreated) as well as fixed in 10% formalin or 1% glutaraldehyde and embedded in paraffin or Epon, respectively. Five-micrometer paraffin sections were subjected to hematoxylin and eosin (H&E), Masson trichrome, and immunohistochemical analyses. Epon-embedded sections (40-nm) were prepared for electron microscopy, with and without osmium and uranyl acetate staining. For immunohistochemical analysis primary mouse anti-human L- or H-ferritin monoclonal antibodies LF03 (1:50) and rh02 (1:1000) respectively, were used (a kind gift of Paolo Santambrogio and Paolo Arosio, Milan, Italy). The control antibody was anti-influenza hemagglutinin of the same isotype (IgG2a) used at a 1:50 dilution. Rabbit anti-mouse IgG sera conjugated to avidin was used as a secondary reagent. Horseradish peroxidase coupled to biotin tertiary reagent was developed with diaminobenzidine.

Transmission electron micrographs of the thin sections of cataract were obtained by a transmission electron microscope (TEM; model 2000; JEOL, Tokyo, Japan) operating at 80 keV. The magnification of the microscope was calibrated by measuring images of tobacco mosaic virus (TMV) stained with uranyl acetate. The TMV sample was supported on a carbon-coated copper grid, which was mounted in the microscope alongside the grid supporting the cataract thin section. The images of the TMV were collected under microscope settings identical with those used to measure the cataract images. Images were digitized to produce 2048 × 2048 eight-bit gray-scale images. The fast Fourier transforms of the digitized images were calculated to identify characteristic length scales translation vectors within the images.

RNA Electrophoretic Mobility Shift Assay

A 289-nucleotide amplicon containing the first 180 nucleotides of the human L-ferritin 5′ UTR was prepared as described by Girelli et al. and cloned into the pCR2.1 vector containing T7RNA polymerase promoter (Invitrogen, San Diego, CA). In vitro transcripts were generated for the sense and antisense strands of wild-type DNA, and the sense strand of the C33T mutant DNA in the presence of [35S]CTP. Binding reactions including 105 counts of the in vitro transcribed RNA and 50 µg protein from cytoplasmic extracts10 were incubated for 10 minutes at room temperature followed by treatment with RNase T1. Cytoplasmic extracts were prepared from the human lens epithelial cell line HLEB51 and from normal, primary, human fibroblasts (from the America Type Culture Collection, Manassas, VA). Parallel binding reactions were performed on cytosolic extracts, with and without 2-mercaptoethanol, to assess total and active IRP activity, respectively. The binding reactions were electrophoresed on nondenaturing 10% acrylamide-ethanol, to assess total and active IRP activity, respectively. The bind-

RESULTS

A Novel HHCS Mutation

Members of a family cosegregating hyperferritinemia and cataract were screened for mutations in the L-ferritin IRE by PCR amplification and DNA sequencing. A novel heterozygous mutation, C33T, was found in the DNA that replaces the unpaired cytosine in the stem of the IRE with uracil (C33T in DNA) in the L-ferritin mRNA (not shown). This change was found in all five affected family members and was absent in an unaffected family member. Analysis of 130 control chromosomes (cataract-free individuals) did not detect the C33T change. An exclusively wild-type IRE sequence was found in all control specimens.

IRP Activity in Human Lens Epithelial Cells and the Effect of the C33U Mutant IRE

To determine whether the C33U mutation of the L-ferritin IRE has functional consequences on IRP binding and on cataract formation we sought to demonstrate that IRP exists in human lens cells. The RNA electrophoretic mobility shift is the assay of choice for IRP function, because IRP bound to IRE-containing RNAs shifts the RNAs in gel electrophoresis. A cytosolic extract from normal human primary fibroblasts and a lens epithelial cell line (HLEB5), as a source of IRP activity, were incubated with radioactive probe from the human L-ferritin 5′ UTR. Arrow: position of a band indicating slow-migrating and nondegraded probe, only when the input RNA is of a sense orientation and therefore capable of forming an IRE hairpin. Sense-strand RNA probes of wild-type and mutant (C33U) sequences are compared for their ability to bind IRP and produce a gel shift. As shown in both lanes, labeled mutant, neither fibroblast (F) nor lens (L) cell IRP did not bind to and retard migration of the mutant RNA probe, because of decreased affinity. This results in the probe’s being degraded.

Crystalline-Appearing Deposits in HHCS Lens

In HHCS, cataracts consist of numerous, small, punctate opacities predominantly in the cortex of the lens, as shown in Figure 3A. Clinically, these cataracts cause progressive glare and impair visual acuity. Light microscopy of a fresh HHCS lens specimen demonstrated frequent, regular, polyhedral, crystalline-appearing deposits (Fig. 3B). These deposits diffract light, as evidenced by the diffraction lines adjacent to the long axis of a deposit (Fig. 3B, arrows). H&E staining revealed uniform cosinophilia of the deposits (Fig. 3C) which also showed strong staining with Masson trichrome (not shown). Congo red produced only background staining of deposits (not shown). De-
was faint anti-L-ferritin immunoreactivity in a minority of lens dense immunoperoxidase reactivity of the deposits (Fig. 4B). There clonal antibody LF03, directed against human L-ferritin, produced comparable titer revealed no staining of the deposits (Fig. 4A). Mono-
tonation of deposits in HHCS lens. Control antibody (isotype-matched) at

Immunostaining was performed to determine the antigenic composi-
tion of deposits in HHCS lens. Control antibody (isotype-matched) at
non-HHCS cataracts (not shown).

FIGURE 3. Features of cataracts and crystalline deposits in HHCS lens. (A) Slit lamp photograph of HHCS eye. Numerous small opacities of the lens are apparent as ‘flecks’ in retroillumination. (B) Crystalline-appearing deposit in HHCS lens aspirate with a typical polygonal appearance. Arrows: diffraction lines outside and parallel to the long axis of the crystal from diffraction of light. No such crystals were observed in four lens aspirates from eyes without HHCS prepared in an identical manner. (C) H&E stained, paraffin-embedded section of lens aspirate, showing a 150-μm-long crystalline deposit. Note the uniform eosinophilia of the deposit and surrounding lens fibers. (D) Transmission electron micrograph (×4000) of a crystalline deposit stained with uranyl acetate and osmium. An electron-dense central area was surrounded by somewhat less dense periphery. Frequent, electron-lucent defects traverse the deposit. These defects were typically linear, ran parallel to an outer face of the crystalline deposit, and intersected at 60° angles. Scale bars, (A, B) 8 μm; (C) 10 μm; (D) 6.25 μm.

positors typically measured 15 to 20 μm wide by 40 to 150 μm long. Low-magnification TEM of deposits demonstrated linear external borders with a dense central area surrounded by a less-dense periphery (Fig. 3D). Prominent ultrastructural features of these deposits were linear defects, or areas of low electron density, the longest of which traversed the deposit (Fig. 3D). These defects had nonrandom orientation and were typically linear and parallel to an exterior face of the deposit (Fig. 3D). Therefore, HHCS lens contained deposits that had histologic staining characteristics of protein with physical and morphologic properties, suggesting a highly ordered structure. No such geometric-appearing deposits were observed in four lens specimens removed and processed under identical conditions from patients with non-HHCS cataracts (not shown).

L-Ferritin in Crystalline- Appearing Deposits in HHCS Lens

Immunostaining was performed to determine the antigenic composi-
tion of deposits in HHCS lens. Control antibody (isotype-matched) at
comparable titer revealed no staining of the deposits (Fig. 4A). Mono-
clonal antibody LF03, directed against human L-ferritin, produced dense immunoperoxidase reactivity of the deposits (Fig. 4B). There was faint anti-L-ferritin immunoreactivity in a minority of lens fibers outside the deposits (Fig. 4B). Anti-H-ferritin monoclonal antibody (rH02) staining of HHCS lens gave a pattern that was indistinguishable from that of control antibody (not shown). Both this anti-H-ferritin and

the LF03 anti-L-ferritin monoclonal antibodies were strongly reactive with human liver under identical conditions (not shown). Therefore, the deposits contained levels of H-ferritin below the limit of immuno-
detection, or the H-ferritin epitope was not accessible to the antibody in the deposit.

Rabbit sera raised against human holoferritin were used to stain sections prepared for immunoelectron microscopy. Preimmune sera produced low-background staining of all lens tissue, including deposits (Fig. 4C). By contrast, electron-dense areas of deposit were strongly reactive with anti-ferritin sera (Fig. 4D). As observed at the light microscopic level there was sparse anti-ferritin staining of lens tissue outside the deposits (not shown). Together, these immunostaining results demonstrate that the crystalline-appearing deposits in HHCS lens were rich in the L-ferritin protein that was overexpressed in HHCS and contained little H-ferritin.

Crystalline Structure and Composition of HHCS Lens Deposits

Because iron is electron dense, ferritin-bound iron may be observed by the electron microscope. Indeed, this has formed the basis for the use of ferritin-conjugated antibodies as markers in immunoelectron micros-
copy for many years. Therefore, unstained (metal-free) electron micros-
scopic sections containing the ferritin-rich deposits were examined, and no electron dense material suggestive of iron was observed (not shown). Electron-dispersive spectroscopy is capable of detecting met-
als in biological specimens. Application of this technique to lens deposits did not detect an iron signal above background, suggesting that the ferritin in lens deposits is not iron rich (data not shown). To investigate structure within HHCS lens deposits, TEM and fast Fourier transformation analysis of the resultant images was performed on a stained section of cataract tissue. TEM revealed regions within the lens deposit that show regular, periodic variations in image contrast (Fig. 5A). Fast Fourier transformations of these regions clearly indicated periodicity in at least two directions (Fig. 5B), indicating a macromo-

FIGURE 4. Anti-ferritin immunostaining of HHCS lens aspirate. (A) A crystalline deposit in HHCS lens aspirate stained with an isotype-
matched, control antibody (anti-influenza hemagglutinin) at 1:50. (B) Anti-L-ferritin (LF03) antibody staining (1:50) revealed dense staining (brown pigment) of two crystalline deposits and little staining of surrounding lens fibers. Note that lens fibers appear to course around the deposits in (A) and (B), so that the deposits disrupt close, parallel packing of lens fibers. (C) Preimmune rabbit sera gave a low back-
ground in immunoelectron microscopy of lens deposit. (D) A crystalline deposit stained with rabbit anti-human ferritin serum followed by anti-rabbit IgG conjugated to gold particles and visualized by electron microscopy. The gold marker strongly stained the electron-dense areas of the deposit. Scale bars, (A) 10 μm; (B) 20 μm; (C, D) 0.5 μm.
Figure 5. High-power TEM and fast Fourier transformation of a deposit confirmed crystal structure. (A) Transmission electron micrograph of a thin section from a region within the crystalline deposit that exhibited periodicity in electron density. (B) The central part of the fast Fourier transform of the image in (A). There were four spots of intensity in addition to the central spot, indicating periodicity shown in (A), in at least two directions, thus confirming the presence of a crystal. Scale bar, 100 nm.

Lectular crystalline structure of these lens deposits. The fast Fourier transforms were consistent with the plane of the thin section's being close to the 110 direction of a face-centered cubic crystal system with a unit cell dimension of 17 nm. Thus, the TEM data in conjunction with the immunostaining results indicate that the lens deposits were composed of crystals of L-ferritin (which is known to crystalize in a face-centered cubic form with a unit cell dimension of approximately 17 nm$^{12,13}$).

Discussion

Iron-dependent regulation of mRNA translation efficiency or stability is a paradigm for posttranscriptional regulation of gene expression in eukaryotes. Two trans-acting factors, IRP1 and IRP2, allow cells to regulate gene expression in response to variation in the intracellular labile iron pool. Iron-responsive elements in ferritin and other mRNAs are the essential cis-elements of this regulatory system. Detailed site-directed mutagenesis of the loop and unpaired cytosine in the stem of the IRE show that most substitutions of six highly conserved residues decrease affinity of IRE-IRP binding.$^{14}$ For example, mutation of the unpaired cytosine (located in the stem of the IRE) to uridine (corresponding to the C33T mutation in the HHCS family reported herein) resulted in a 29-fold decrease in affinity for IRP in an RNA gel shift assay. Further suggestion that the bulge region in the stem of IRE is important comes from the fact that mutation of G32 (the nucleotide immediately preceding C33) to either A or T has been found in different HHCS families (data not shown and Ref. 15). The naturally occurring C33T mutation, reported herein for the first time, disrupts IRP binding and causes HHCS. This naturally occurring human mutation confirms the importance of the unpaired cytosine and bulge region in the IRE stem by showing that mutant IREs disrupt IRP-dependent regulation of L-ferritin expression in vivo.

Two mechanisms were proposed to explain the etiology of cataract in HHCS. The first was that overexpression of L-ferritin disrupts iron homeostasis—for example, that a change in the L-to-H subunit ratio of ferritin composition may increase free iron and reactive oxygen species with concomitant oxidative damage to lens. However, in keeping with the fact that L-ferritin does not bind iron directly, Levi et al.$^{3}$ found no evidence of increased iron in the lens of patients with HHCS. Herein, direct evidence that there is no increase in iron derives from our analysis of L-ferritin crystals in HHCS lens. The iron level is below the limit of detection by electron-dispersive spectroscopy (not shown).

The second postulated mechanism invokes a well-known cause of cataract formation: loss of lens protein solubility. By this hypothesis, the overexpressed ferritin directly forms deposits or insoluble aggregates that are lens opacities. Ferritin-rich deposits with the appearance of crystals have been reported to be cataracts in HHCS.$^{9}$ We have extended this analysis to demonstrate that the cataracts are light-diffracting crystals composed of iron-poor, L-ferritin-rich, and H-ferritin-poor isoform. The evidence is as follows: First, deposits were found in HHCS lenses that are 3 to 10 lens fiber diameters across (Fig. 3C). This size was sufficient to disrupt packing of lens fibers (Figs. 4A, 4B) and was predicted to disrupt light transmission through the lens. Second, these deposits had a striking geometric (i.e., polyhedral) appearance (Figs. 3B–D, 4A, 4B). Third, internal linear defects in the deposits were geometric and nonrandom in structure (Fig. 3D). Fourth, the crystalline appearance of deposits diffracted light (Fig. 3B). Fifth, polyclonal and monoclonal antibodies against human ferritin densely stained the lens deposits (Fig. 4). Sixth, periodicity was evident in at least two directions in the deposits, as demonstrated by fast Fourier transformation of transmission electron micrographs (Fig. 5), indicating crystalline structure. The fast Fourier transform is consistent with a face-centered cubic crystal structure that is one of the crystal structures adopted by purified ferritin crystallized in vitro.$^{12,13}$ Finally, the 17-nm unit cell size in this crystal is consistent with that of isoform crystals grown in vitro with an intermediate size between that expected for a wet isoform ferritin crystal (18.4 nm) and that of an air-dried ferritin crystal (15.8 nm)$^{12,13}$ Together, these data confirm the presence of L-ferritin crystals in HHCS lens. We argue that L-ferritin crystals are the lens opacities that cause HHCS cataracts, because these crystals both diffract light and disrupt lens fiber packing and therefore focusing of light.

Cataract formation is the most prominent and only consistent clinical abnormality in HHCS. Because the gene is ubiquitously expressed, dysregulation of L-ferritin expression in HHCS is predicted to occur in most if not all tissues. Indeed, overexpression of L-ferritin in HHCS has been demonstrated in serum,$^{1,2}$ liver,$^{4}$ lymphoblasts$^{5}$ and the eye (aqueous and vitreous humors and lens).$^{3}$ Why might L-ferritin overexpression have a propensity to affect lens more than other tissues? At increased concentration, L-ferritin may cause cataracts because of properties of lens physiology that may promote protein crystallization. First, there is a very high-protein concentration in lens. Second, there is little protein turnover after formation of mature lens fibers. Lens proteins have half-lives measured in decades. Third, lens is avascular and surrounded by a dense...
capsule; therefore, ferritin synthesized within the lens may not diffuse or be diluted out. Thus, when abnormally high L-ferritin concentrations are produced in HHCS lens, conditions such as time, low protein turnover, and high protein concentration are likely to contribute to ferritin crystallization. These features of lens biology may account for the absence of reported phenotypic changes in other tissues.

Crystal formation in the path of light transmission through the eye is inherently problematic, due to light diffraction. A single case of crystal formation in lens, as a sole cause of cataract, was reported previously,16 but the substance of the crystals remains unknown. Crystallization of calcium oxalate in lens has been reported in association with complicated cataract.17 Recently, protein crystals have been found to be cataracts for the first time. Specifically, two different mutations in human lens crystallin genes cause crystalline cataracts.18,19 Unlike HHCS, wherein the crystallized L-ferritin is of wild-type sequence, the proteins causing crystalline cataract in these instances are mutant. In one case, the mutant amino acid creates a new salt bridge proposed to stabilize crystal structure.18

Analysis of lens removed from two patients with HHCS during cataract surgery has provided important insight into the basis of cataract in this disease. The HHCS lens contains frequent crystals that are sufficiently large to disrupt packing of lens fibers and therefore the focusing of light by the lens. Subunits of these crystals have the size, crystallization characteristics, and antigenic properties of L-rich isoferritin. Physical appearance and properties, such as polyhedral shapes and periodic electron density within the deposit, demonstrate the crystalline nature of these L-ferritin deposits. This is the third example, to our knowledge, of protein crystals as a cause of cataract and may well represent the most common example, because more than 50 families with HHCS are known (data not shown). Future research will address the prevalence of HHCS, natural history of crystal formation, the implications of ferritin insolubility outside the eye in HHCS and other diseases, and potential therapeutic measures for crystal cataract diseases such as HHCS.

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References


